



# Autopsy Report: A Case of *Vibrio vulnificus* Infection

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## Abstract

The infection caused by *Vibrio vulnificus* can develop a course of disease quickly and has high mortality. The high risk population of the infection are people with immune system, especially those having chronic, long term alcoholic, or suffering from hematochromatosis. The bacterial laboratory at Taiwan CDC received 11 autopsy samples from a patient suspected infection by an infectious pathogen. The condition of the case progressed worst rapidly on the same day at admission. The laboratory identified the bacteria isolated to clarify any bacterial pathogen related to the course of disease. The results showed that only one bacterial pathogen, Gram-negative, curved, rod-shaped, isolated from the heart-blood swab. The pathogen was also identified from the menigeal swab, pericardial effusion swab, and pleural effusion swab. After identification by biochemical kits and traditional biochemical reaction, the pathogen was confirmed as *Vibrio vulnificus*. To use a proper and strict sterile sampling

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technique for autopsy samples from a case suspected of infection by an infectious pathogen can reduce sample contamination, which is the main factor influencing or confusing the judgment effectively. To collect suitable samples at a proper time point can avoid the influence caused by many normal flora either translocated into blood or samples lysed. Hence, the investigation and the analysis for - an illness developed quickly, such as an infection caused by *Vibrio vulnificus* can be effective. Moreover, results based on an accurate and meaningful bacterial examination can be achieved.

**Keywords:** *Vibrio vulnificus*, septicemia, autopsy case, biochemical identification

## Introduction

*Vibrio vulnificus* is a halophilic bacterium, Gram-negative, curved, and rod-shaped, which grows properly on warm sea-water. Its natural habitat is the environment near sea-bays. Most of infectious cases are reported from the seashore of the Pacific Oceans, American, and European countries along the coast [1]. The first *Vibrio vulnificus* infectious case reported in Taiwan was from Kaohsiung area in 1985. After that, many cases have been diagnosed on clinic. Recently, the trend of getting infection by the pathogen has increased dramatically. Most cases were reported from Southern Taiwan. So far, more than 200 cases have been reported. Because the patient's condition is usually severe and the course of the disease develops fast, clinical infection control doctors have kept an eye on the pathogen since the first case was reported [2-4]. Two main syndromes related to the infection are primary septicemia and wound infection. For the first one, the infection route is incurred after eating contaminated raw or under-cooked seafood, such as fish, shrimps, and



oysters. The symptoms, such as fever, chills, anorexia, vomiting, diarrhea, pathological changes on the skin, pain on limbs' skin, necrotic ulcers, and septic shock, start after 16 hours of infection. The mortality can be as high as 50%. Most of patients died within 48 hours of admission. For the condition of wound infection, patients got infection because their open wounds contact with contaminated seawater, seafood, or the patients are punctured by shrimps, or crabs. The skin infection shows reddish, swelling, and blistering vesicles within 12 hours of infection, then necrotic cellulites developed later. Antimicrobial agents and surgical trials are necessary in this condition. The ulcerative area must be removed; in some condition, amputation may be necessary to avoid the severe progress of the disease from the wound. For more severer cases, secondary septicemia is expected. The mortality rate is approximately 24% [5-7].

The risk factors of *Vibrio vulnificus* infection are patients with compromised immune system. Usually, patients with chronic diseases, especially patients with liver dysfunction, cirrhosis, chronic renal failure, hemochromatosis, alcohol abuse for a long time, and diabetes mellitus, are highly to develop severe symptoms. Fifty percent of patients infected have history of eating seafood, or have exposure history of seafood or sea-water [5,8]. There are many reports related to the study of pathogenesis. Although according to the reports, the capsule of *Vibrio vulnificus* and the mechanism of intaking the iron ions via transferring and lactoferrin are the main pathogenic reasons, the true pathogenesis and the roles of pathogenic factors in *Vibrio vulnificus* infection are still unclear. Chuang YC et al., are very zealous to study the therapeutic effect of different drugs and proved that the combination of cefotaxime and minocycline have good prognosis in

treating the infection. The protocol has been acknowledged and used widely internationally [11,12]. However, because the course of disease develops quickly and the high mortality of the infection, the important and efficient prevention policies should avoid to contact with the possible sources of infection for patients with chronic diseases and potential risk factors, to diagnose accurately on time at clinic, and an aggressive therapy, including vigorous antimicrobial treatments, surgery, and supportive treatments.

The bacterial laboratory at Taiwan CDC received a suspected case infected by infectious pathogen on 3, Sep, 2007. The case was a 54 years old male and lived in Da-Ya Town, Taichung County. His chief complaint was painful legs on 22, August; the date he went to a hospital. He was transferred into another hospital because his condition became severe quickly. When the time he arrived to the emergency department, he had lost his consciousness. He died three hours later because of cardiac, pulmonary and renal failures. The examination results in hospital showed pulmonary infiltration, rhabdomyolysis, renal failure, hyperkalemia, inflammatory indexes rising, and *Vibrio cholera* was isolated from the case's blood sample on 28, August. The CDC performed the autopsy and collected samples on 31, August. To clarify any possible bacterial pathogen related to the acute development of disease, laboratory used bacterial isolation culture and other identification methods to analyze possible pathogenic bacteria from the autopsy samples. This report also stated the sampling property and the examination protocol in the laboratory to provide information for doctors and staffs of epidemic prevention and control.



## Materials and Methods

### 1. Samples

Eleven samples were collected from this autopsy; these are the meningeal swab, the lung swab, the heart-blood swab, the pericardial swab, the pleural swab, the bile swab, the small intestinal swab, the large intestinal swab, the lung tissue, the aerobic and the anaerobic blood bottles. The laboratory performed examinations after receiving the samples.

### 2. Bacterial isolation and identification

The swabs were inoculated into the selective and differential agars according to the nature of the samples (Table 1); included the blood agar plate (BAP), the chocolate agar, the MacConky sorbitol agar, the SS agar, the TCBS agar, the anaerobic BAP and the PEA. Tissue samples were put into sterilized buffered solution and then ground by grinder. A certain amount of sample solution was inoculated into the suitable agars selected. The blood bottles were first inoculated into agars when received. The bottles were placed in a blood bottle incubator (BD, NJ, USA) and then the growth was observed for the following 7 to 10 days. If there was any sign of growth in this period, the sample was inoculated into an agar again. When there is no sign of growth the sample was inoculated into an agar one more time to confirm it was a negative result. The BAP and the chocolate agar were incubated in an environment at 37°C and at CO<sub>2</sub> of 5%. The MacConky sorbitol agar, the SS agar, and the TCBS agar were cultured at 37°C. The anaerobic BAP and the PEA were placed in an anaerobic jar at 37°C; the condition was produced by a gas-pak system. The incubation time was more than 48 hours for the chocolate agar and the anaerobic BAP, other cultures were observed for 18 to 24 hours.

**Table 1. The agars used for inoculation and isolation in this case<sup>a</sup>**

Types of samples	Agars <sup>b</sup>					
	BAP	M-S	SS	TCBS	Chocolate	ana.BAP/PEA
meningeal swab	✓	✓			✓	✓
lung swab	✓	✓			✓	
heart blood swab	✓	✓		✓	✓	
pericardial swab	✓	✓		✓	✓	
pleural effusion swab	✓	✓		✓	✓	
bile swab	✓	✓		✓		
small intestinal swab	✓	✓	✓	✓		
large intestinal swab	✓	✓	✓	✓		
lung tissue	✓	✓			✓	
blood in aerobic bottle	✓	✓		✓	✓	
blood in anaerobic bottle	✓			✓	✓	✓

<sup>a</sup> The case's samples were inoculated into suitable agars according to the purposes of isolation.

<sup>b</sup> BAP: the blood agar plate; M-S: the MacConky sorbitol agar; SS: the *Salmonella Shigella* agar; TCBS: the thiosulfate citrate bile salts sucrose agar, Chocolate: the chocolate agar; ana.BAP/PEA: the anaerobic blood agar plate and the anaerobic phenylethyl alcohol agar.

### 3. The observation of colonial morphology and Gram's staining

The colonies' types, distribution and the ratio of colonies on agars were observed first. Secondly, each single colony's morphology, such as shape, size, color, characteristics from the surface and the margins, and the characters of hemolysis were observed. Then, different colonies selected on agars were observed using Gram stain and were re-cultured for the purpose of the continuing identification. If too many colonies grew on the agars and then the numbers of colonies were not able to be counted, the main colonies were identified only; on the contrast, if only few colonies grew on, they were all selected for identification. If the colonies only grew on the first or the second areas on the agars, or the numbers of colonies were able to be counted, all different colonies were selected for identification. If only one or two different morphological colonies were



found on the agars, the colonies were treated as contaminated bacteria and were not worth of identification. Gram-Hucker's stain solution (Muto Pure Chemical Co., LTD, Tokyo, Japan) was used. A certain amount of bacteria was smeared on a slide and then fixed. The results after staining were observed under oil immersion lens.

#### **4.The bacterial biochemical identification**

For bacteria of Gram's stain (+) bacteria, the catalase reaction was tested firstly. If it was positive, the coagulase reaction was performed as follows. The oxidase reaction was used for Gram negative bacteria. According to the reaction of Gram's stain and the following biochemical reactions, a suitable biochemical identification kit was chosen. Basically, Vitek (bioMerieux, Marcy-I'Etoile, France) and Phoenix (BD) Automated Microbiology System and its reagent kit was used to identify the colony. If a special or an uncommon on clinic was found or atypical reaction was recorded, a more suitable commercial kit would be used. After the identification procedures, to re-confirm the examination result, a series of traditional biochemical tests and tests helpful in re-confirmation were executed to isolate the pathogen. In this case, its response of the biochemical reactions and the result of the salt-tolerance were listed on Table 3.

#### **5.The identification of bacterial 16S rDNA**

The important pathogenic bacteria, the special pathogen on clinic, and the bacteria with atypical biochemical reactions isolated in the case would amplify their 16S rDNA sequences in order to reconfirm the results of identification. MicroSeq (Applied biosystems, CA, USA) was used to identify the bacteria based on the 16S rDNA sequence. The QIAamp DNA mini kit (Qiagen, Hilden, Germany) was used to purify the selected

bacterial DNA. The 16S gene targeted was amplified using the PCR technique, in which in total reaction solution of 30  $\mu$ L contained 15  $\mu$ L of 2X master mix, 5  $\mu$ L of DNA template, 10  $\mu$ L of sterilized water. The Biometra PCR reaction machine was used to amplified the templates according to the condition that was denature at 95°C for 10 minutes, followed by 35 cycles of denature at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 45 seconds, and then 72°C for 10 minutes. After the above reaction, the product was shown in an agarose gel to confirm the size of the product. After purification of the target DNA fragment, the product was then proceeding for nucleotide sequence. The sequence of the 16S rDNA was analyzed by MicroSeq ID Analysis Software 2.0 (Applied Biosystems) and BLAST (BLAST <http://www.ncbi.nlm.nih.gov/>).

## **Results**

### **1.The characteristics of colonial morphology and Gram's staining**

It helps to identify and judge the bacteria under microscopy that to observe the colonies' morphological characteristics on agars after inoculation and after Gram's stain. To observe the growth of colonies, such as its types, distribution, and ratio on agars, helps judge and identify bacterial clinical meanings. The observation of colonies' growth was listed on Table 2. The bacterial colonies isolated from the case's meningeal swab, heart-blood swab, and bile swab grew the same morphological colonies. The colonies from the pericardial effusion swab and pleural effusion swab were pure; only two different morphological colonies were observed. The colonies grew on above agars were all selected and identified. Samples





from others, including the blood bottles, the agar medium was fully covered with many different morphological colonies. Hence, only the main colonies were identified. The colonies grew on the BAP and which isolated from the patient's meningeal swab, heart-blood swab, pericardial effusion swab, and pleural effusion swab showed brownish, smooth and creamy characteristics. In addition, beta-hemolysis was observed on the colonies on these agars. The characteristics of the colonies from the bile swab on the agars were very different; the colonies showed they were purely milk-like and of non-hemolysis. The bacteria selected were stained by Gram's solution. Most of the bacteria were Gram's negative rods, the bacteria isolated from the meningeal swab, the heart-blood swab, the pericardial effusion swab, and the pleural effusion swab was curved and short-rod-like. Few bacteria isolated were Gram's positive cocci, including bacteria selected from the lung swab, the lung tissue, the pericardial swab, and the aerobic and the anaerobic blood bottles.

## **2.The identification of the bacteria isolated**

According to the rule listed above, the results of identification for the bacteria selected were listed on Table 2. *Vibrio vulnificus* was cultured and identified from the meningeal swab and the heart-blood swab, in which the same morphological colonies were cultured. It was also identified from the pneumopericardial swab and the pleural effusion swab. *Escherichia coli* was identified from the bile swab. From the aerobic and anaerobic blood bottles, the bacteria identified were considered as normal flora in bodies; *Vibrio vulnificus* was not isolated. The other bacteria identified from the pneumopericardial swab and the pleural swab were all identified as normal flora of a body and did not show any clinical meaning.

**Table 2. The results of the bacterial isolation and identification from the case's samples<sup>a</sup>**

Samples	Bacterial isolation and identification	
	The results of colonial growth	Identified results of the isolated bacteria
meningeal swab	About 50 same morphology.	<i>Vibrio vulnificus</i>
lung swab	Flora grew fully Full growth on agar. Two main morphological colonies were observed. Few other morphological colonies grew as well <sup>b</sup>	<i>Klebsiella pneumoniae</i> , <i>Escherichia coli</i> , <i>Streptococcus spp.</i>
heart-blood swab	About 20 colonies grew with same morphology.	<i>Vibrio vulnificus</i>
pericardium swab	About 20 colonies grew; two morphological colonies were observed.	<i>Vibrio vulnificus</i> , <i>Streptococcus spp.</i>
pleural effusion swab	About 40 colonies grew with two morphological types.	<i>Vibrio vulnificus</i> , <i>Escherichia coli</i>
bile swab	About 200 colonies with same morphology.	<i>Escherichia coli</i>
small intestinal swab	Colonies grew fully on the agar; two main morphological colonies were observed.	<i>Escherichia coli</i> , <i>Klebsiella pneumoniae</i>
large intestinal swab	Colonies grew fully on the agar; two main morphological colonies were observed.	<i>Escherichia coli</i> , <i>Klebsiella pneumoniae</i>
lung tissue	Colonies grew fully on the agar; two main morphological colonies were observed. Few other morphological colonies were observed as well <sup>b</sup>	<i>Klebsiella pneumoniae</i> , <i>Escherichia coli</i> , <i>Streptococcus spp.</i>
The aerobic blood bottle	Colonies grew fully on the agar; three main morphological types.	<i>Klebsiella pneumoniae</i> , <i>Escherichia coli</i> , <i>Streptococcus spp.</i>
The anaerobic blood bottle	Colonies grew fully on the agar; three main morphological types.	<i>Klebsiella pneumoniae</i> , <i>Escherichia coli</i> , <i>Streptococcus spp.</i>

<sup>a</sup> Different morphological colonies on agars were selected for identification according to the rules: different morphological colonies were selected and identified if only a kind and two kinds of colonies grew on; main colonies were selected and identified if the colonies grew on covering the plate fully; all colonies were selected and identified if few colonies grew on.

<sup>b</sup> Few colonies were identified as *Streptococcus spp.*

### 3. The identification and isolation of *Vibrio vulnificus*

After the bacteria were identified and isolated from some of the case's samples, *Vibrio vulnificus* was isolated from the heart-blood swab and it was the only one grown from the swab. It was also isolated from other samples and was considered as a significant pathogen with clinical meanings. To reconfirm the result, the traditional biochemical

characteristic reactions and other examinations beneficial to the identification were performed. The results were listed on Table 3. The bacteria isolated from the TCBS were greenish, middle-sized and smooth colonies. It presented brownish, smooth and creamy colonies with beta-hemolysis on the BAP. The result of the traditional biochemistry reaction was oxidase (+). The score and the identification result was *Vibrio vulnificus* (94%) from the Viteck biochemical test kit was; *Vibrio vulnificus* (99%) from the Phoenix test kit; and *Vibrio vulnificus* (99%) from 16S rDNA bacterial analysis. It did not grow in 0% NaCl of 0% but grew in 6% NaCl in the salt-tolerance test. The other characteristics are lysine decarboxylase (+), and arginine dihydrolase (-). The above information showed that the species of the bacteria identified from the most samples was *Vibrio vulnificus*.

**Table 3. The identified characteristics of *Vibrio vulnificus* from the case<sup>a</sup>**

Observation and examination	Observation and reaction
Colonial morphology-TCBS	Middle size (2-4 mm), greenish, smoothy
Colonial morphology-BAP	Middle size (2-4 mm), brownish, smoothy creamy
Type of hemolysis	β-hemolysis
Gram's stain	Gram's negative curve flex rods
Oxidase	Positive
Viteck	<i>Vibrio vulnificus</i> 94%
Phoenix	<i>Vibrio vulnificus</i> 99%
16S rDNA	<i>Vibrio vulnificus</i> 99%
Salt-tolerance	Growth: 3% NaCl , 6% NaCl Non-growth: 0% NaCl , 8% NaCl
The traditional bio-chemical characters	Positive: ONPG, indole, motility, lysine, glucose, maltose Negative: H <sub>2</sub> S, VP, citrate, ornithine, arginine, urea, malonate, adonitol, arabinose, inositol, rhamnose, sorbitol, sucrose

<sup>a</sup> Related confirmation were performed for the pathogenic bacteria isolated with clinical meaning

## Discussion

The infection of *Vibrio vulnificus* is likely to occur in chronic liver disease and immunocompromised patients. There are 80 times higher chance of getting severe symptoms in this kind of patients, compared with ordinary people. In Taiwan, there is a high prevalence of chronic liver disease. In addition, Taiwan is an island surrounding by sea and has developed fishery industry. Thus, Taiwanese have higher chance to get infection by contacting with the pathogen. For the higher risk population, especially those who have a contact history, *Vibrio vulnificus* should be suspected when special skin symptoms or sepsis is developed. The BAP and the TCBS agars are useful to isolate *V. vulnificus*, in which greenish, middle-sized, smoothy and creamy colonies will grow on the TCBS agar or beta-hemolysis will present on the BAP agar. *Vibrio vulnificus* is oxidase (+), sucrose (-), arabinose (-), indole (-), motility (+), VP (-), and lysine (+). It does not grow on salty water of 0% to 8%. It can be identified accurately and on time based on its biochemical reaction characteristics.

Morris JA et al. investigated the principle of aetiological judgment based on the results of bacteriology from autopsy samples. In addition, the clinical meanings and the values of the results were also discussed [13]. Five thousands bacterial examination results from autopsy cases were evaluated. The bacteria identified could be from 4 situations: 1) They are the true sources of pathogens causing the infection; 2) They have attacked tissues or samples from organs before the patient dies due to the distribution of pathogens of non-caused of death; 3) They translocate into blood or organs from the surface of mucosa (postmortem translocation); 4)



They are isolated because of contamination. Their results presented that the most important confoundings or the reasons confusing the results are the samples' contamination. Moreover, when the autopsy is performed, a suitable, conscientious and careful aseptic technique, following aseptic procedure exactly, decontaminated equipments, and avoiding cross-contamination by change sampling tools are able to reduce the appearance of contaminated bacteria. In addition, collecting the samples at the proper time point, e.g. within 24 hours of death, or keeping the case at 4°C before autopsy can avoid the normal flora in a body fluid into blood or samples; then contaminated bacteria can be avoid to be isolated from samples. Only one pathogenic pathogen growing on agars from the samples of the blood or the brain-spinal fluid means that it is the most possible pathogen resulting in the infection, and deserves for further identification and confirmation. Then, it is worth of confirm and identification deeply. Both the evidence from the clinical history and the histopathological examination of the histological change must be considered and matched to get the truly clinical meaning. To get the final diagnosis based on aetiology would be difficult if there are too many bacterial flora grown on the sample collected from the autopsy. The study suggested that analyzing properly and judging some confoundings would support true and meaningful results from the examination of bacteriology or investigate bacterial infectious diseases efficacy [13,14].

Our laboratory isolated and identified and isolated *Vibrio vulnificus* from this case. The species of bacteria is the only one grown flora from his the heart-blood swab. It was also identified from the patient's meningeal swab, the pericardial effusion swab, and the pleural effusion swab. The

pathogenic pathogen was not able to be identified from the aerobic and anaerobic blood bottles even though there were multiple growths; although in which many flora had grown on. The possible reason might due to the is that the normal flora common bacteria in a body have translocated into blood massively or the samples had been contaminated, and then the results of isolation and identification were interfered. The case was an alcoholic with cirrhosis and usually went to seashore to pick up conches to earn a living; it showed that he had a history of contacting with seafood and sea water. The symptoms developed rapidly and fatal to death quickly with rhabdomyoysis and acute renal failure on the day at admission that the case sought doctor's advice. According to the above information and his clinical signs, the cause of death could be thought to be related to the infection of *Vibrio vulnificus*. The other bacteria were all considered as normal flora in a body and as non-pathogenic pathogens without any clinical meanings.

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